Coexpression of Fragile Histidine Triad and c-kit Is Relevant for Prediction of Survival in Patients with Small Cell Lung Cancer

Nina Rehfeld,[1] Helene Geddert,[2] Abedelsalam Atamna,[1] Helmut E. Gabbert,[2] Ulrich Steidl,[3] Roland Fenk,[1] Ralf Kronenwett,[1] Rainer Haas,[1] and Ulrich-Peter Rohr[1]

[1]Klinik für Hämatologie, Onkologie und klinische Immunologie and [2]Institut für Pathologie, Heinrich-Heine-Universität Düsseldorf, Dusseldorf, Germany and [3]Harvard Institutes of Medicine and Harvard Stem Cell Institute, Boston, Massachusetts

Abstract

Background: In a retrospective analysis of 195 patients with small cell lung cancer (SCLC), we examined the prognostic value of a coexpression of fragile histidine triad (FHIT) protein and c-kit on patient's survival.
Methods: As assessed by immunohistochemistry using formalin-fixed, paraffin-embedded tissue sections, tumors of 195 patients with SCLC were evaluated for FHIT and c-kit coexpression.
Results: Coexpression of FHIT and c-kit was observed in 53.3%; a positive expression of either FHIT or c-kit was found in 40.5%. Complete lack of FHIT and c-kit (6.2%) was associated with a significantly shorter survival time for the patients with a mean of 122 ± 45 days compared with 468 ± 89 days for patients with lung cancer coexpressing FHIT and c-kit ($P = 0.0011$). The proportion of FHIT- and c-kit-positive cells within a tumor was also related to survival time. Patients with tumors with a proportion between 0% to 25% of FHIT- and c-kit-positive cells had the worst survival of 157 ± 34 days compared with 496 ± 95 days for patients showing >25% FHIT- and c-kit-positive cells ($P = 0.0002$). Further, variables associated with shorter survival times were low performance status, elevated lactate dehydrogenase level, and advanced tumor stage according to tumor-node-metastasis classification. Multivariate analysis using Cox regression model, including 11 variables, confirmed the prognostic significance of a combined expression of FHIT and c-kit next to tumor stage, performance status, and lactate dehydrogenase level.
Conclusions: Differential FHIT and c-kit expression was of prognostic relevance for survival in patients with SCLC and therefore provide useful variables for therapeutic decisions. (Cancer Epidemiol Biomarkers Prev 2006;15(11): 2232–8)

Introduction

Recently published data suggest that the fragile histidine triad (FHIT) protein and the tyrosine kinase c-kit are involved in the pathophysiology of small cell lung cancers (SCLC). The loss of the *FHIT* gene, located on 3p14.2, has been proposed to be involved in the development of lung cancer. The 18.8-kDa FHIT protein consists of 146 amino acids and is a human orthologue of the fission yeast *Schizosaccharomyces pombe* protein, a diadenosine $5',5\text{-}P_1P_4$-tetraphosphate hydrolase (1). Although the exact molecular pathway of FHIT signaling is unknown, the FHIT substrate complex seems to be involved in the regulation of p53-independent apoptosis (2, 3) and cell cycle control (4). Molecular analysis has shown that between 90% and 100% of SCLC and between 50% and 80% of non-SCLC have a loss of heterozygosity on chromosome 3p encoding for FHIT (5, 6). Further, loss or down-regulation of FHIT in lung cancer is associated with hypermethylation of the FHIT promoter (7, 8). Looking at 19 primary SCLCs and 28 SCLC cell lines, only between 20% and 47% of the tumors were FHIT positive (9-11). In a previous study, we immunohistochemically examined 225 patients with SCLC and observed a loss of FHIT expression in 38.2% of the patients, which was significantly associated with unfavorable prognosis (12).

The *c-kit* gene, located on 4q12, encodes for a tyrosine kinase growth factor receptor for stem cell factor, also known as CD117 or c-kit (13). Autocrine and paracrine activation of growth-stimulatory signaling plays an important role in the development of SCLC. The potential role of c-kit in the carcinogenesis of SCLC is suggested by *in vitro* findings showing that increased proliferation and migration of SCLC cells can be induced, when c-kit-positive cell lines are exposed to the stem cell factor, which is the physiologic ligand for c-kit (14-17). In addition, growth of SCLC cell lines is inhibited by antisense-mediated suppression of c-kit receptor (18). At the moment, the reports are somehow inconsistent and the function of c-kit has been associated with both favorable prognosis (19) and unfavorable prognosis (20, 21). According to published data, between 27.9% (19) and 78% (20) of SCLC express c-kit (20, 21). In our retrospective analysis, the SCLC patients with c-kit-positive tumors had a significant better prognosis compared with the patients with c-kit-negative tumors (22). In an attempt to clarify the role of FHIT and c-kit as biomarkers in patients with SCLC, we examined the influence of a coexpression of the two genes in 195 patients on survival. For that purpose, univariate and multivariate analyses were done.

Patients and Methods

Characteristics of the Patients with SCLC. Formalin-fixed, paraffin-embedded tissue sections from 195 patients with primary SCLC were used for immunohistochemical detection of FHIT and c-kit. Specimens were obtained from the Institute of Pathology of the University of Dusseldorf (Dusseldorf, Germany). Biopsies from the primary lung tumor were taken

at initial clinical presentation. The original diagnosis of SCLC was confirmed by two different pathologists before the biopsy was accepted for this study. The histopathologic diagnosis was based on H&E stains in each case and confirmed by immunohistochemical staining of cytokeratin, chromogranin A, and synaptophysin. The patients enrolled in the study were patients from the University of Dusseldorf and associated academic hospitals of the University of Dusseldorf. Clinical data of the patients were collected from chart review with given approval from the ethics committee of the University of Dusseldorf. Baseline characteristics of chosen patients are presented in Table 1. Survival time in days was calculated from the date of histopathologic diagnosis. Performance status of the patients was evaluated by applying standard WHO criteria. All tumors were classified according to the current tumor-node-metastasis system (23). The stages I to IV were based on the revised version of the International System for Staging Lung Cancer (24). Chemotherapy was done as a first-line treatment with an average of 3.11 cycles (median, 3.0 ± 2.2 cycles; range, 1-11) in 79.2% of all evaluable SCLC patients, respectively. The preferred chemotherapeutic regimen for 57% of SCLC patients was a combination of cyclophosphamide (1,000 mg/m^2 dL), epirubicin (65 mg/m^2 dL) or Adriamycin (45 mg/m^2), and etoposide (120 mg/m^2). A platinum-based combination with cisplatin (90 mg/m^2) or carboplatin (300 mg/m^2) and etoposide (150 mg/m^2) was given in 12.8%, whereas other combinations were given in 9.4% of patients. For 6.2% of the patients, surgical resection was preferred. Patients (37.4%) received additional radiotherapy of the primary tumor or of the brain.

Immunohistochemistry. Briefly, fresh tumor tissue specimens were formalin-fixed and paraffin-embedded. Tissues were cut in 2- to 4-μm sections, deparaffinized in xylene, and rehydrated through increasing concentrations of ethanol. Deparaffinized sections were subsequently preincubated in 3% hydrogen peroxide for 15 minutes followed by incubation in a blocking solution (DAKO, Hamburg, Germany) for 15 minutes to prevent nonspecific binding. Next, the slides were incubated with anti-FHIT and anti-c-kit antibody for 1 hour. A polyclonal antibody raised against full-length human FHIT (Zymed, San Francisco, CA) was used for standardized immunohistochemical staining procedure as described by Ramp et al. (25) As described previously by Micke et al. (20) and Naeem et al. (21), a standardized immunohistochemical staining procedure was used for c-kit detection, using a primary polyclonal antibody against c-kit that targets the COOH-terminal domain of the intracellular part of the c-kit receptor (DAKO). After washing, a streptavidin horseradish peroxidase detection kit (DAKO) containing 3,3′-diaminobenzidine solution as substrate was used for immunohistochemical staining according to the manufacturer's instructions. For c-kit detection, gastrointestinal stromal tumor tissue and normal lung tissue served as positive and negative control, respectively. For FHIT detection, human kidney was used as a positive control. All slides were simultaneously assessed by two investigators. In a final control process, all slides were reviewed and the results were confirmed by a third investigator (H.E.G.). The staining intensities for FHIT and c-kit were evaluated semiquantitatively and classified into three groups. The first group showed equal or stronger cytoplasmic staining intensity compared with positive control. The second group showed lower staining intensity of FHIT or c-kit compared with the positive control. The third group showed no immunohistochemical evidence of FHIT or c-kit expression. For the estimation, all tumor areas of one to four biopsies from bronchoscopy were included. The proportion of positive cells was determined semiquantitatively: (*a*) none, (*b*) between 1% and 25%, (*c*) between 26% and 75%, and (*d*) >75% of the tumor cells with FHIT or c-kit expression.

Statistical Methods. Kaplan-Meier survival analysis with log-rank test was used to compare clinical variables with FHIT/c-kit expression. Two-sided *t* test was done to compare clinical variables of FHIT/c-kit–positive and FHIT/c-kit–negative group. For multivariate analysis, a Cox regression model with a forward stepwise selection was used. The following variables were included for multivariate analysis: gender (male versus female), age (≤60 versus >60 years), performance status (classified into WHO 0/I versus II/III), tumor stage (WHO classification), hemoglobin level (<12 versus ≥12 mg/dL), platelet count (<150,000/μL, 150,000-400,000/μL, and >400.000/μL), lactate dehydrogenase (LDH) level (classified into serum levels, ≤240 versus >240 units/L), leucocyte count (≤11,000/μL versus >11,000/μL), smoking status (smoker versus nonsmoker), as well as staining intensity of FHIT/c-kit coexpression and quantitative FHIT/c-kit coexpression. Pearson's bivariate correlation was done to evaluate a correlation between FHIT/c-kit-coexpressing cells to clinical variables. Statistical analysis was done using SPSS software 12.0. Significance is defined as $P < 0.05$ and the respective values are given in the text.
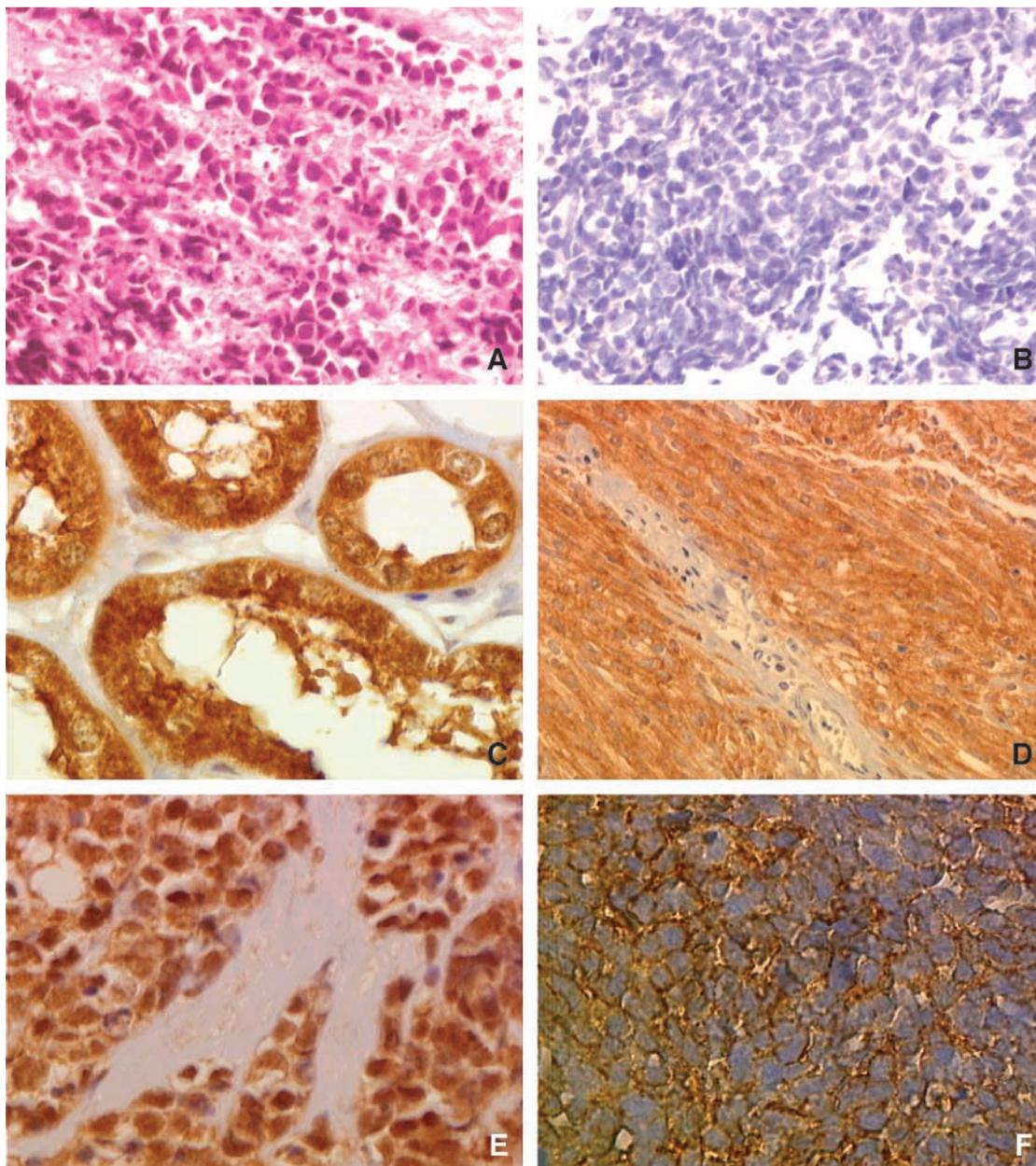
Results

Immunostaining of FHIT in Tumors from Patients with SCLC. FHIT expression was observed in 116 of 195 (59.5%) examined tumors. With regard to the degree of expression, low cytoplasmic staining intensity was found in 65 (33.3%) cases, whereas strong cytoplasmic staining intensity was found in 51 (26.2%) cases, respectively (Fig. 1).

In most FHIT-positive tumors, the proportion of FHIT-expressing cells was above 75% (76 of 195, 39%). Proportion of FHIT-positive cells between 1% and 25% and between 26% and 75% was found in 6 (3.1%) and 34 (17.4%) of all tumors, respectively, whereas 79 (40.5%) tumors were FHIT negative.

Immunostaining of c-kit in Tumors from Patients with SCLC. c-kit expression was observed in 171 of 195 (87.7%)

Table 1. Baseline characteristics of patients with SCLC (*N* = 195)

	Patients (%)
Mean age (range), y	62.8 ± 9.7 (40.4-87.4)
Sex	
Male	141 (72.3)
Female	54 (27.7)
Smoking status	
Smoker/former smoker	127 (65.1)
Nonsmoker	22 (11.3)
Not evaluable	46 (23.6)
WHO performance status	
0	55 (28.2)
I	72 (36.9)
II	17 (8.7)
III	7 (3.6)
IV	1 (0.5)
Not evaluable	43 (22.1)
Stage	
Ia,b	5 (2.6)
IIa,b	10 (5.1)
IIIa,b	37 (19)
IV	92 (47.2)
Not evaluable	51 (26.1)
LDH (units/L)*	380 ± 360
Hemoglobin (g/dL)*	13.75 ± 1.86
Thrombocytes (μL)*	324.292 ± 110.688
Leukocytes (μL)*	$9,752 \pm 3,500$

*Mean $\pm$ SD.

Figure 1. Immunohistochemical staining of FHIT and c-kit. **A.** H&E staining of SCLC. **B.** SCLC with negative FHIT expression. **C.** FHIT-positive control of uropoietic tubuli of the kidney. Uropoietic stromal cells between the tubuli were FHIT negative. **D.** c-kit-positive control of a gastrointestinal stromal tumor. **E.** SCLC with strong FHIT expression. **F.** SCLC with strong c-kit expression.

examined tumors. Looking at staining intensity of c-kit expression, low cytoplasmic staining intensity was found in 93 (47.7%) cases and strong cytoplasmic staining intensity was found in 78 (40%) cases (Fig. 1).

Similar to FHIT staining, majority of c-kit-positive tumors showed a positive staining of >75% of the cells (86 of 195, 44.1%). Proportion of c-kit-positive cells between 1% and 25% and between 26% and 75% were found in 7 (3.6%) and 78 (40%) of all tumors, respectively, whereas 24 (12.3%) tumors were c-kit negative.

Coexpression of FHIT and c-kit in Tumors from Patients with SCLC. Lack of FHIT and c-kit was observed in 12 (6.2%) of all 195 patients with SCLC. The expression of only one protein, either FHIT or c-kit, was found in 79 of 195 (40.5%) SCLCs. On the other hand, a coexpression of FHIT and c-kit was observed in 104 of 195 (53.3%) tumors.

Prognostic Value of FHIT and c-kit Expression in Patients with SCLC Using Kaplan-Meier Survival Curves. Median and mean survival rates for all 195 patients with SCLC were 201 ± 18 days and 347 ± 49 days, respectively. This translates into 1- and 5-year survival rates of 25.8% and 2.9%, respectively. Looking at FHIT expression, a significant improved survival rate was observed for those patients with positive FHIT expression compared with those with negative FHIT expression. Patients without FHIT expression had a significant ($P = 0.0092$) shorter mean survival of 216 ± 30 days compared with 436 ± 80 days for those patients with positive FHIT expression (Fig. 2A).

Looking at patients with or without c-kit expression, a significant better survival ($P = 0.0078$) was observed for the patients with positive tumors, who had a longer mean survival of 372 ± 55 days. In comparison, the survival was only

154 ± 28 days for those patients with negatively stained tumors (Fig. 2C).

Looking at the proportion of FHIT-positive tumor cells first, the patients with FHIT-negative tumors or with a FHIT-expressing proportion ≤25% had a significantly poorer prognosis with a mean survival of 206 ± 29 days compared with 454 ± 83 days for patients with tumors showing a FHIT-positive cell proportion >25% (*P* = 0.0014; Fig. 2B).

Turning to the influence of c-kit-expressing tumor cell proportion patients with negative c-kit expression or c-kit-expressing tumor cells ≤25% had a significant shorter mean survival of 167 ± 24 days compared with 379 ± 58 days for patients with a c-kit-positive cell proportion higher than 25% (*P* = 0.0065; Fig. 2D). No significant difference was observed

between the patient groups with respect to gender, age, smoking status, performance status, tumor stage, mean cycles of chemotherapy, mean hemoglobin level, mean platelet count, mean leukocyte count, and mean LDH using two-sided *t* test.

Prognostic Value of FHIT and c-kit Coexpression in Patients with SCLC. To analyze the prognostic influence of a coexpression of FHIT and c-kit compared with the presence of one receptor or the lack of both receptors in SCLCs, three groups were formed: group I, patients with no FHIT and c-kit expression; group II, patients with one positive protein, FHIT or c-kit; and group III, patients with a coexpression of FHIT and c-kit. Patients showing an expression of both proteins had a better survival of 468 ± 89 days compared
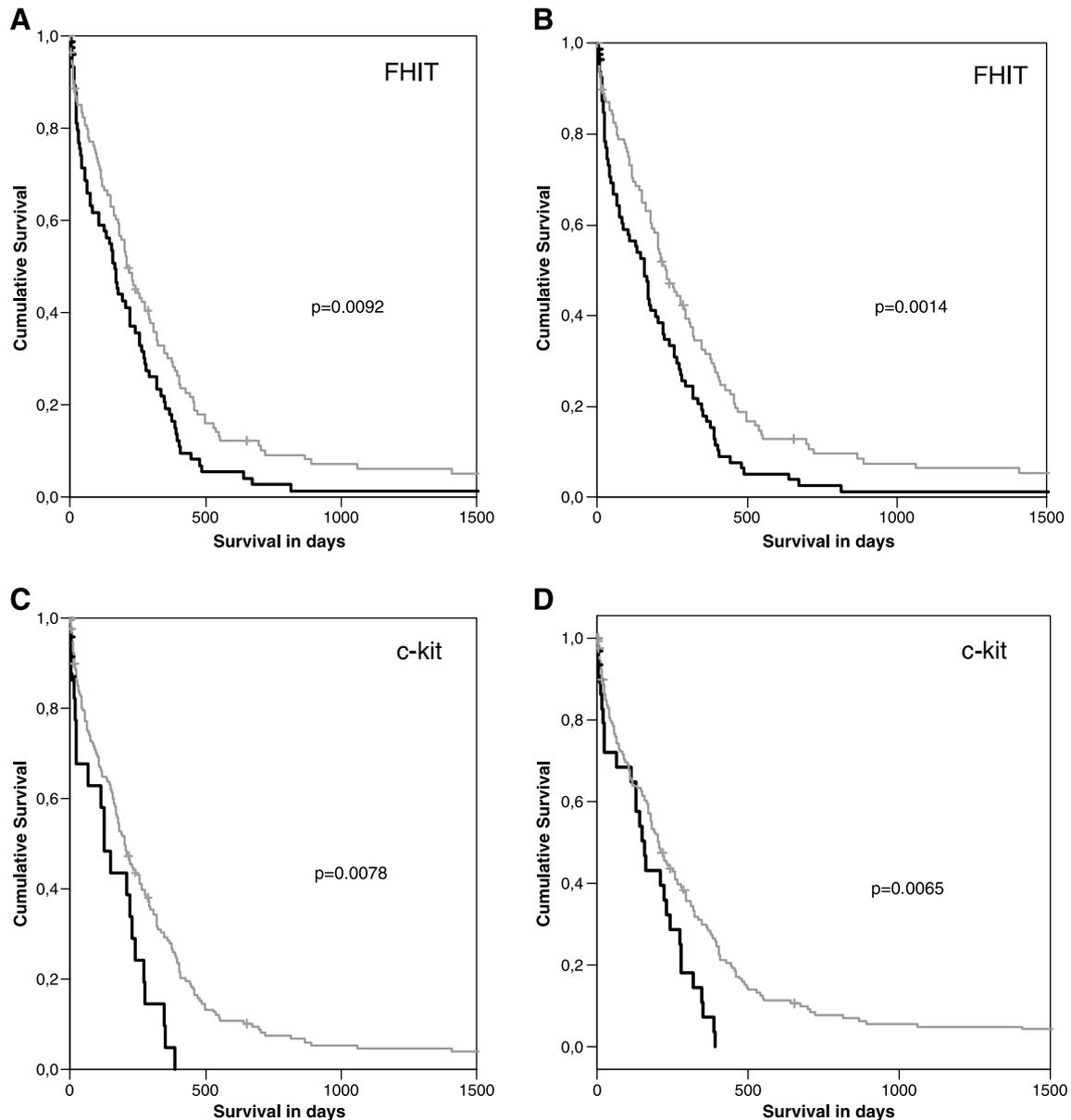
Figure 2. A. FHIT expression and patient's survival. Lack of FHIT expression (*black line*) was significantly associated with poorer survival compared with tumors with positive FHIT expression (*gray line*). **B.** Proportion of FHIT-expressing tumor cells and patient's survival. Survival was significantly reduced for patients with FHIT-positive cells ≤25% (*black line*; including negative cases) compared for those tumors showing a FHIT-positive cell proportion >25% (*gray line*). **C.** c-kit expression and patient's survival. Lack of c-kit expression (*black line*) was significantly associated with poorer survival compared with tumors with positive c-kit expression (*gray line*). **D.** Proportion of c-kit-expressing cells and patient's survival. Survival was significantly reduced for patients with c-kit-positive cells ≤25% (*black line*; including negative cases) compared for those tumors showing a c-kit-positive cell proportion >25% (*gray line*).
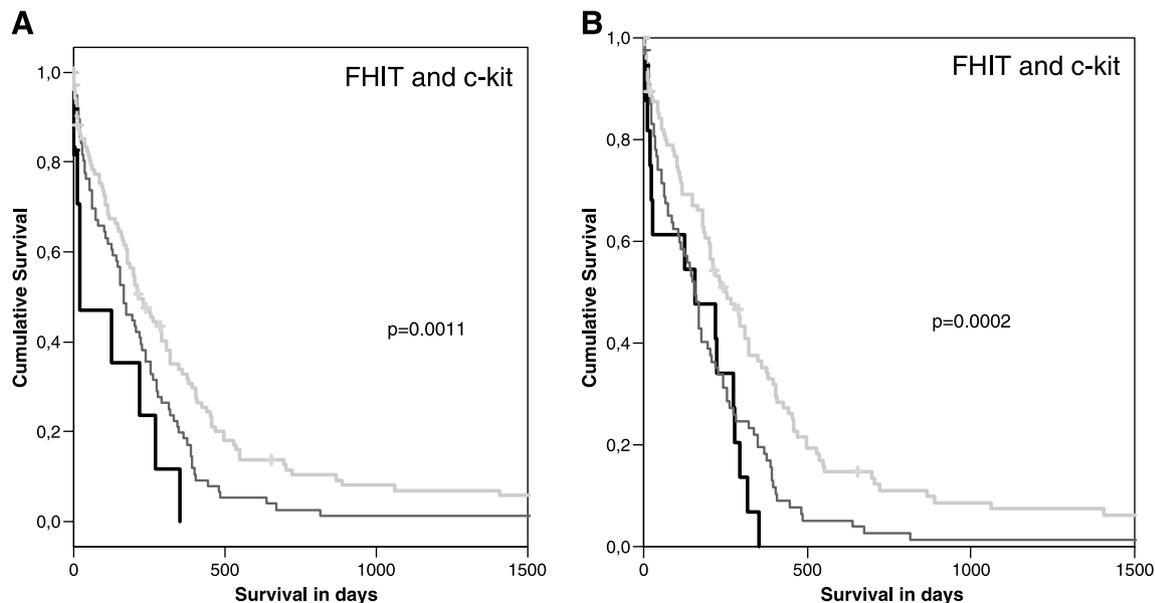
Figure 3. A. Association of FHIT and c-kit coexpression and survival. Lack of FHIT and c-kit (*black line*) was associated with a significant shorter mean survival compared with patients with either FHIT- or c-kit-positive cells (*dark gray line*) or compared with patients with a coexpression of FHIT and c-kit (*light gray line*). **B.** Association of the proportion of FHIT- and c-kit-positive cells within a tumor and survival. A proportion of FHIT- and c-kit-positive cells ≤25% (*black line*; including negative cases) was associated with a significantly reduced survival compared with tumors showing at least an expression of one receptor >25% (*dark gray line*) or compared with FHIT- and c-kit-positive cells >25% (*light gray line*).

with 222 ± 29 days for the patients showing at least expression of FHIT or c-kit. Patients showing neither FHIT- nor c-kit-expressing tumor cells had the worst prognosis with a mean of 122 ± 45 days ($P = 0.0011$; Fig. 3A).

Next, the proportion of tumor cells expressing FHIT and c-kit was correlated to survival. To evaluate the proportion of FHIT and c-kit expression in SCLC, three groups were formed. Patients in group I with an expression ≤25% of FHIT and c-kit had the worst prognosis with a mean survival of 157 ± 34 days compared with 496 ± 95 days for patients in group III showing a coexpression of FHIT and c-kit >25% ($P = 0.0002$). Patients in group II with at least an expression of one receptor (FHIT or c-kit) >25% had an intermediate prognosis with a survival of 210 ± 29 days (Fig. 3B). All three groups did not show statistical differences with respect to variables, such as patient's gender, age, smoking status, performance status, tumor stage, mean cycles of chemotherapy, mean hemoglobin level, mean platelet count or mean leukocyte count, or mean level of LDH.

Clinical Variables and FHIT/c-kit Coexpression in Multivariate Analysis for Patients with SCLC. Next to FHIT or c-kit status, other clinical variables, such as performance status (WHO 0/I versus II/III; $P < 0.0001$), tumor stage (I versus II versus III versus IV; $P = 0.0024$), and LDH level (serum levels, ≤240 versus >240 units/L; $P = 0.0067$), were also related to survival time using Kaplan-Meier analysis. According to Pearson's bivariate correlation analysis, FHIT/c-kit coexpression was not correlated to any other clinical variables. To test FHIT/c-kit coexpression as an independent prognostic factor, multivariate analysis was done. Consequently, the proportion of FHIT and c-kit coexpression ($P = 0.006$), tumor stage ($P = 0.005$), performance status ($P = 0.013$), and LDH level ($P = 0.036$) was identified as independent prognostic variables by the Cox regression model. In contrast, thrombocyte count, leucocyte count, hemoglobin level, gender, smoking status, and age were irrelevant with regard to survival time ($P > 0.05$; Table 2).

Discussion

In this study, we retrospectively examined FHIT and c-kit coexpression in 195 patients with SCLC tumors and its implication for prognosis. Expression of FHIT was observed in 59.5% of 195 SCLC tumors as already shown previously (12). Patients with complete lack of FHIT had a significantly shorter survival compared with patients with tumor cells expressing FHIT. This might be related to the function of FHIT as a tumor suppressor gene. In this line, 53% of homozygous FHIT-deficient mice developed spontaneous tumors within 2 years compared with 8% of animals expressing wild-type FHIT (26). Furthermore, gene transfer experiments with adenoviral vector-mediated replacement of wild-type FHIT (Ad-FHIT) in lung cancer cell lines that lacked endogenous *FHIT* gene expression led to a significant reduced cell growth up to 80% (27). In accordance to these studies and similar to our results, Toledo et al. (28) recently examined 98 primary non-SCLC with regard to FHIT expression and found a poorer survival for the patients with

Table 2. Variables accepted in the forward selection model of the Cox regression as explanatory factors in SCLC patients

	P
Proportion of FHIT/c-kit coexpression*	0.006
Stage[†]	0.005
Performance status[‡]	0.013
LDH[§]	0.036

*Tumors showing a cell proportion of FHIT and c-kit between 0% and 25% (group I), tumors showing a cell proportion of one protein (FHIT or c-kit) >25% (group II), and tumors showing a coexpression of FHIT and c-kit in >25% of the tumor cells (group III).
[†]According to tumor-node-metastasis system: stage I/II versus III/IV.
[‡]Performance status according to WHO: 0/I versus II/III.
[§]LDH serum levels, ≤240 versus >240 units/L.

FHIT-negative tumors. Further, Tomizawa et al. (29) had also seen a significantly improved survival for the patients when the tumor was FHIT positive looking at 105 patients with non-SCLC. These reports underline the role of FHIT as a tumor suppressor gene in lung cancer patients.

The tyrosine kinase c-kit (CD117) is known to play an oncogenic role in various tumors, including gastrointestinal stromal tumors (30), mast cell disease (31), and chronic myeloproliferative disorders (32). In our patients, an expression of c-kit was observed in 87.7% of 195 SCLC tumors as shown previously (22). The patients with tumor cells expressing c-kit had a significant longer survival time compared with patients with tumor cells lacking c-kit expression. At a first glance, our result indicating a better survival for those patients with c-kit expression seems to be paradoxical because one would expect a growth advantage of c-kit-expressing tumor cells mediated by an autocrine loop between the c-kit receptor and its ligand SCF (15, 33) leading to an inferior survival. A possible explanation might be that c-kit-positive SCLC cells are more susceptible to cytotoxic treatment because more tumor cells are in active cell cycle. Consistent with this view are the results of a report of Tamberi et al. (34) showing that untreated patients with SCLC were c-kit positive at time point of diagnosis but negative when relapsing after a cytotoxic chemotherapy. This suggests that the recurrent tumor might be derived from a population of c-kit-negative tumor cells. Consequently, in a phase II trial, the tyrosine kinase inhibitor imatinib (Glivec), which targets the intracellular domain of the c-kit receptor and inhibits the downstream signaling, was not effective in relapsed patients with SCLC (35).

Focusing on the prognostic value of a FHIT and c-kit coexpression in our subgroup of 195 patients with SCLC tumors, we could show that patients with tumors lacking FHIT/c-kit had the worst prognosis with a mean survival of 4 months compared with 7 months for patients with tumors being positive for at least one protein. The patients with tumor cells showing a coexpression of FHIT and c-kit had the most favorable prognosis with a mean survival time of 15 months. Furthermore, the proportion of FHIT- and c-kit-positive tumor cells with a cutoff level of 25% within a tumor had an influence on survival. Patients with FHIT- and c-kit-negative tumor cells or a tumor cell proportion ≤25% had the worst prognosis showing a mean survival of 5 months compared with 16 months for patients with a FHIT- and c-kit-coexpressing tumor cell proportion >25%. Patients with a tumor cell expression >25% of at least one of the two proteins had an intermediate prognosis with a mean survival of 7 months. This result was confirmed by multivariate analysis using the Cox forward selection model to identify independent prognostic factors.

The mean survival difference of 11 months between FHIT/c-kit–negative and FHIT/c-kit–positive coexpressing groups is surprisingly high, considering the overall mean survival time of patients with SCLC of 6 to 10 months. Our data underline the role of FHIT and c-kit as relevant genes in the pathophysiology of lung cancer and their role as possible biomarkers predicting individual prognosis for patients with SCLC. In our study, we showed a significant improved survival for patients with a combined expression of FHIT and c-kit in the SCLC tumor cells compared with a single protein expression (FHIT or c-kit) or compared with a negative FHIT and c-kit expression. Our results also imply a synergistic effect of FHIT and c-kit coexpression on SCLC patient's survival, which can be taken into contemplation for the individual therapy with regard to dose intensification or consideration for high-dose chemotherapy followed by autologous stem cell transplantation. A prospective study is now envisaged to corroborate the prognostic significance of FHIT and c-kit on stage- and treatment-adapted patient's survival. At best, two molecular biomarkers are available for the future for an individualized therapy with possible dose intensification in dependence of the biomarker profile.

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Nina Rehfeld, Helene Geddert, Abedelsalam Atamna, et al.

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